

CG

⑬  **Europäisches Patentamt**
European Patent Office
Office européen des brevets

⑪ Publication number:

0 142 299
A2

⑫ **EUROPEAN PATENT APPLICATION**

⑲ Application number: 84307289.3

⑤① Int. Cl.⁴: C 12 Q 1/68

⑳ Date of filing: 23.10.84

Priority: 25.10.83 JP 199702/83

④③ Date of publication of application:
22.05.85 Bulletin 85/21

① Designated Contracting States:
CH DE FR GB IT LI NL

⑦① Applicant: FUJIREBIO KABUSHIKI KAISHA also trading
as FUJIREBIO INC.
6-7, Shimoochiai 4-chome Shinjuku-ku
Tokyo 161(JP)

⑦② Inventor: Ashihara, Yoshihiro
2-402, Fuchudanchi 28-1, Harumicho, 1-chome
Fuchu-shi Tokyo(JP)

⑦② Inventor: Kasahara, Yasushi
310, 4-4, Nagayama 3-chome
Tama-shi Tokyo(JP)

⑦④ Representative: Silverman, Warren et al,
HASLTINE LAKE & CO. Hazlitt House 28 Southampton
Buildings Chancery Lane
London WC2A 1AT(GB)

⑤④ Method of measuring polynucleotide and reagent kit for use therein.

⑤⑦ Disclosed is a method of measuring a polynucleotide such as DNA and RNA in a body fluid which does not require immobilisation of the test sample and whereby the polynucleotide is easily determined, wherein an immobilised single-stranded polynucleotide which is labelled is hybridised with the single-stranded polynucleotide to be measured, the double-stranded polynucleotide formed by hybridisation is broken by means of a restriction enzyme and the labelling substance present is then determined as a measure of the polynucleotide being determined.

EP 0 142 299 A2

1 "METHOD OF MEASURING POLYNUCLEOTIDE AND REAGENT KIT FOR
FOR USE THEREIN"

This invention relates to a method of measuring polynucleotide, i.e. deoxyribonucleic acid (DNA) or
 5 ribonucleic acid (RNA), in a fluid sample, in particular a body fluid.

The measurement of a DNA in a sample of human serum provides significant results when testing for a viral infection or a hereditary disease. A conventional
 10 method of detecting a particular DNA comprises denaturing of a double-stranded DNA (d-DNA) in a sample under investigation to produce a single-stranded DNA (s-DNA), immobilising the s-DNA on a carrier material, hybridising the immobilised s-DNA with a radioisotope-labelled s-DNA,
 15 removing unreacted radioisotope-labelled s-DNA, and then measuring radioactivity of the immobilised material. Since this method comprises many processes including immobilisation of each sample, it is complicated to carry out and hence the procedure is time consuming.

20 It is an object of this invention to provide a simpler method of measuring a polynucleotide.

According to this invention, there is provided a method of measuring a polynucleotide which is either single stranded as such or a single stranded
 25 polynucleotide obtained from a double stranded polynucleotide to be measured, which comprises, contacting the single-stranded polynucleotide to be measured with an immobilised labelled single-stranded polynucleotide which is able to hybridise with said
 30 single-stranded polynucleotide in aqueous solution to produce a double-stranded polynucleotide, breaking the double-stranded polynucleotide thereby obtained by means of a double-stranded polynucleotide restriction enzyme, and measuring the amount of labelling substance in the
 35 solution obtained and/or on immobilised material, as appropriate as a measure of said single stranded and/or said double stranded polynucleotide to be measured.

1 This invention also provides a reagent kit for use
in said method comprising the aforesaid immobilised
labelled single stranded polynucleotide and a restriction
enzyme.

5 The method of this invention uses as starting
material a single-stranded polynucleotide, hereinafter
termed s-polynucleotide to be measured, which may be
derived from a double stranded polynucleotide in which
case its quantity is a measure of the polynucleotide from
10 which it has been obtained. The s-polynucleotide to be
measured may be s-DNA or s-RNA. When the fundamental
polynucleotide in a sample to be determined is
double-stranded, it is necessary that the polynucleotide
be denatured by alkaline treatment using a sodium
15 hydroxide solution or heating to convert it to
single-stranded polynucleotide. Test materials which may
be subjected to this method include human serum, urine
and tissue extracts. When a protein is combined with
the polynucleotide in a sample, such as serum, the
20 protein is preferably separated off by using, for
example, a protease.

The immobilised single-stranded polynucleotide
(labelled s-polynucleotide) incorporates or has bound
thereto a labelling substance and is able to hybridise
25 with the s-polynucleotide to be measured. Hence, this
labelled s-polynucleotide acts as a probe which can
hybridise with the s-polynucleotide to be measured. The
s-polynucleotide to be labelled may be produced by
denaturation of a double-stranded polynucleotide
30 containing the s-polynucleotide to be measured by means
of alkaline treatment or heating. It may also be
produced by a known solid phase polynucleotide synthesis
method or by a genetic synthesis method accomplished with
 γ -DNA using a plasmid. Some s-polynucleotides have, in
35 themselves, found commercial use.

The labelling substance which is used may be a
radioisotope, a fluorescent material, a luminescent

1 material, a magnetic material, an enzyme, a prosthetic
group of such an enzyme, a coenzyme, or an enzyme
inhibitor or activator. Radioisotopes which may be used
include ^{32}P , ^3H , ^{35}S , ^{14}C and ^{125}I . Fluorescent
5 materials which may be used include fluorescent reagents,
such as rhodamine, fluorescein, methylcoumarin and dansyl
chloride. Luminescent materials which may be used
include chemically luminescent materials such as
isoluminol and luminol, and a biologically luminescent
10 material such as luciferine-luciferase. Enzymes, for
example glucose oxidase, peroxidase and alkaline
phosphates, whose activities can easily be measured are
preferably used. Since processes for measuring a
polynucleotide include heating, the enzyme is preferably
15 thermostable. Should an enzyme which is not
thermostable be used, hybridisation may also be carried
out at 35 to 40°C. Prosthetic groups include flavine
adenine dinucleotide which is an active site of a glucose
oxidase, and its amount can be determined by measuring
20 the activity of a holoenzyme after reaction with an
apoenzyme. Coenzymes which may be used, include NADH,
NADPH₂, aminopyrophosphate, pyridoxal phosphate, ADP and
ATP.

The carrier material of the labelled
25 s-polynucleotide may be Sepharose, Sephadex (Registered
Trade Marks), cellulose gel, ion-exchange resin, filter
paper, nitrocellulose, nylon, polystyrene or
polyacrylamide.

Immobilisation may be carried out by following a
30 general immobilisation method or a general polynucleotide
synthesis route. In such a procedure, an
oligonucleotide is first allowed to bind to a carrier
material, and then, the s-polynucleotide is allowed to
bind to the oligonucleotide by using a 3'-nucleotidase.
35 An amino group or carboxyl group is introduced to the
s-polynucleotide, and then it is allowed to bind to a
carrier material having carboxyl groups or amino groups

1 by using a reagent for peptide synthesis such as
dicyclohexyl carbodiimide or carbonyldiimidazole. When
both the s-polynucleotide and a carrier material have
amino groups, they can be combined by using for example
5 cyanuric chloride or glutaraldehyde. When both the
materials contain SH groups, they can be combined by
using a reductant or a reagent having maleimide group.
A physical adsorption method may also be employed for the
immobilisation. For example, nitrocellulose is immersed
10 in a solution of the s-polynucleotide for a prescribed
time and then washed. Finally, the immobilised product
may also be produced by connecting nucleotides
successively on a carrier material using a solid phase
synthesis method.

15 Introduction of the labelling substance into the
s-polynucleotide may be carried out by following a
conventional method. The introduction is usually
carried out prior to the immobilisation. However, when
the labelling substance is a large molecule like an
20 enzyme, the introduction is preferably carried out after
the immobilisation.

The s-polynucleotide to be measured is contacted
with the labelled s-polynucleotide in the solution phase.
The contacting time is usually about 0.5 to 40 hours.
25 The temperature of the solution is preferably from 20 to
70°C, and the pH is usually 5 to 9.

The double-stranded polynucleotide restriction
enzyme then used (hereinafter abbreviated as "restriction
enzyme") is preferably one which is specific for
30 double-stranded polynucleotides, and preferably
recognises a short polynucleotide chain. Moreover two or
more different restriction enzymes may be employed. The
restriction enzyme is usually added to the solution after
the hybridisation, although it may be added together with
35 or prior to the s-polynucleotide to be measured.

After the restriction enzyme reaction, the
immobilised material is separated from the solution, if

1 necessary, and the residual labelling substance in the
solution or of the immobilised material, as appropriate,
is measured. The measurement may be carried out by a
known method. For example, radioactivity of this
5 material may be determined by using a scintillation
counter or a Geiger counter in the case of a radioisotope
labelling element.

Characteristically, the method of the invention
does not need the immobilisation of the s-polynucleotide
10 to be measured, and is easy to carry out. Instead,
standard reagents, and optionally devices, can be made
available as a kit. A polynucleotide such as DNA can then
be easily detected or determined by using this kit.

The following Examples illustrate this invention:

15 EXAMPLE 1

(1) Preparation of HBV-DNA Probe

500 ml of a pool serum from chronic B-type
hepatitis patients were centrifuged at 9000 rpm for 15
minutes, and the supernatant was further centrifuged
20 using an ultracentrifuge at 4°C at 100,000 xg for 5 hours
to collect HBV particles as a pellet. This pellet was
dissolved in 10 ml of 0.01 M tris-HCl buffer solution of
pH 7.5 containing 0.1 M NaCl, 1 mM EDTA, 0.1% by weight
2-mercaptoethanol and 0.1% by weight of BSA. 5 ml of the
25 virus solution were stored, and the remaining 5 ml were
centrifuged at 100,000 xg for 5 hours to obtain a pellet
again.

The pellet was treated with 200 µl of 10 mM
tris-HCl 0.1 M NaCl pH 7.5 solution containing 0.5% by
30 weight NP-40, and DNA polymerase was thereby activated.
50 µl of 0.08 M MgCl₂ 0.2 M tris buffer solution of pH
7.5 containing 1 mM dATP, 1 mM dTTP, 2.5 µM ³²PdGTP and 2.5
µM ³²PdCTP were added to this solution which was allowed
to warm up for 3 hours. Then, the solution was layered
35 over 30% by weight sucrose solution placed in a
centrifuge tube, and centrifuged at 50,000 rpm for 3
hours using a SW 65 rotor (made by Beckman Co.) to obtain

1 a pellet. This pellet was treated with a protease, and
two extractions from the solution were carried each with
phenol. The extracts were combined and placed on 5 to
20% by weight sucrose solution gradient, and centrifuged
5 at 50,000 rpm for 3 hours. $15\text{ S}^{32}\text{PDNA}$ fractions were
collected, and pooled. $15\text{ S}^{32}\text{PDNA}$ was precipitated from
the pooled fractions by adding ethanol, and dried to
obtain the target HBV-DNA.

(2) Preparation of DNA Probe by Nick Translation

10 1 μg of the above HBV-DNA was added to 100 μl of
50 mM tris-HCl buffer solution of pH 7.5 containing 5 mM
MgCl₂, 10 mM 2-mercaptoethanol, 5 μM dTTP, 5 μM dGTP, 5 μM
- ^{32}P -dCTP (relative radioactivity 100-700 Ci/mmol) and 5
 μM - ^{32}P -dATP (relative radioactivity 100-700 Ci/mmol).
15 100 pg DNase I and 100 pg DNA polymerase I were added to
the solution which was incubated at 15°C for 90 minutes.
Extraction of radioactive HBV-DNA from the solution was
carried out by using phenol, and the extract was purified
by using a Sephadex G-50 column.

20 1 mg of this HBV-d-DNA (1 ml) was mixed with 5 mg
of single-stranded M13-phage DNA (2 ml). 8 ml of
formamide were added to the mixture which was then boiled
for 5 minutes. Subsequently, 2 ml of 0.07 M tris-HCl
buffer solution containing 2 M NaCl and 15 mM EDTA (pH
25 7.5) were added to the solution which was then warmed at
 50°C for 4 hours and then at 60°C for 1 hour.

The reaction mixture obtained was separated by
gel-filtration using Bio-Gel A50 m, and hybridised DNA
and unreacted DNA were removed. The first peak fraction
30 near the void fraction was collected, and NaCl powder was
dissolved therein in a concentration of 0.1 M. 100%
ethanol in a volume twice that of the solution was added,
and the solution obtained allowed to stand at -70°C for 2
hours. Subsequently, the solution was centrifuged at
35 17,000 xg for 10 minutes, and the precipitates which
formed were collected. The precipitates were dissolved
in 50 ml of 0.001% Phenol Red solution containing 0.1 N

- 1 NaOH and 0.25 mM EDTA, and purified by gel-filtration
using Bio-Gel A50 to obtain the target HBV-s-DNA.

Meanwhile, 1 μ g HBV, 100 pg DNase I and 100 pg DNA
polymerase I were added to 100 μ l of 50 mM tris-HCl
5 buffer solution of pH 7.5 containing 5 mM $MgCl_2$, 10 mM
2-mercaptoethanol, 5 μ MdTTP, 5 μ MdGTP, 5 μ MdCTP, 5
 μ MdATP, 10 μ M aminohexyl dATP and 10 μ M aminohexyl dCTP
and the solution was incubated at 15°C for 90 minutes.
Extraction from the solution was carried out by using
10 phenol, and the extract was purified by using Sephadex
G-50 column to obtain a modified HBV-d-DNA.

This d-DNA was converted to s-DNA by following the
aforementioned procedure.

(3) Preparation of Immobilised Material

15 A polyuracil nucleotide was allowed to bind to
CNBr-activated Sepharose gel beads. The radioactive
HBV-s-DNA was added to the gel beads, and the 3'-terminal
position of the polyuracil was allowed to bind to
5'-terminal position of the radioactive HBV-DNA by adding
20 RNA ligase. The gel beads were sufficiently washed with
50% dimethylformamide containing 0.2 M NaCl to yield the
required immobilised material.

1.0 ml of d-DNA restriction enzyme solution (Bgl
II, Ava II, Hae II, Hae III, Hap II, Hinc II - each 1
25 U/ml, 10 mM tris-HCl, 7 mM $MgCl_2$, 70 mM NaCl, 7 mM
2-mercaptoethanol, pH 7.5) was added to the immobilised
material, and allowed to react at 37°C for 1 hour.
Then, the immobilised material was sufficiently washed
with the same dimethylformamide solution, and HBV-d-DNA
30 was completely removed.

(4) Measurement of Sample

100 μ l of 0.5 N NaOH solution were added to 100 μ l
of the serum of a HB viral hepatitis patient and
stirring at ambient temperature was carried out for 10
35 minutes. Subsequently, the solution was neutralised by
adding 100 μ l of 0.5 N HCl, and 200 μ l of 200 μ g/ml
proteinase K solution were then added. The solution was

1 allowed to react at 70°C for 1 hour. The immobilised materials prepared in step (3) were suspended in 1 ml of the solution. The mixture was allowed to stand overnight at 37°C. The mixture was centrifuged, and the
5 supernatant was removed. 1.0 ml of d-DNA restriction enzyme solution (Bgl II, Ava II, Hae II, Hae III, Hap II, Hinc II - each 1 U/ml, 10 mM tris-HCl, 7 mM MgCl₂, 70 mM NaCl, 7 mM 2-mercaptoethanol, pH 7.5) was added, and allowed to react at 37°C for 1 hour. After the
10 reaction, the resulting mixture was centrifuged, and the radioactivity of 500 µl of the supernatant was measured by using a scintillation counter.

The radioactivity reading was converted to dilution ratio of serum using the previously obtained
15 standard plot of Fig.1 on which the ordinate axis indicates the counting rate of radioactivity, and the abscissa indicates dilution.

EXAMPLE 2

(1) Preparation of Immobilised Material

20 A solution containing 500 µg of aminohexyl-induced HBV-s-DNA was dropped onto a nitrocellulose filter (5 x 5 cm) so as to permeate all over the filter. The filter was dried by heating under reduced pressure. Subsequently, the filter was washed sufficiently with 0.2
25 M NaCl, and immersed in 0.1 M carbonate solution of pH 8.0.

100 mg of N⁶-(6-carboxyhexyl)-adenine flavine dinucleotide were dissolved in 10 ml of dimethylformamide. 50 mg of N-hydroxysuccinimide and 70
30 mg of water-soluble carbodiimide were added to this solution, and reaction was allowed to take place at ambient temperature for 1 hour. After the reaction, this solution was allowed to permeate all over the filter which was allowed to stand at ambient temperature for 2
35 days. This filter was washed sufficiently with 0.1 M carbonate solution, and, in this way, unreacted aminohexyl adenine flavine dinucleotide was removed.

1 (2) Measurement

100 μ l of 0.5 N NaOH were added to 100 μ l of the serum of a HBV viral hepatitis patient, and stirred at ambient temperature of 10 minutes. Subsequently, the solution was neutralised by adding 100 μ l of 0.5 N HCl, and 200 μ l of 200 μ g/ml of proteinase K were then added. The solution was allowed to react at 70°C for 1 hour, and then 200 μ l of saturated phenol-chloroform solution (1:1) were added. 200 μ l of the aqueous layer separated off therefrom were dropped on to the above filter (1 x 1 cm), and allowed to react at 60°C for 2 hours. 1.0 ml of d-DNA restriction enzyme solution (Hae III, Hinc II, Xba I, Bgl II, Ham HI, Ava II, Alu I - each 1 U/ml, 20 mM tris-HCl, 7 mM MgCl₂, 70 mM NaCl, 7 mM 2-mercaptoethanol, pH 7.5) was added, and allowed to react at 37°C for 15 minutes. After the reaction, the filter was removed, and 200 μ l of a substrate solution (0.025% by weight ABTS, 1.8% β -D-glucose, 7 μ g/ml POD, 0.1 M phosphate, pH 6.0) containing 10 μ g of apoglucose oxidase were added. Rate assay was carried out by measuring the increase of absorbance at 420 nm.

The reading obtained was converted to dilution ratio of serum using the previously obtained standard plot of Fig.2 on which the abscissa indicates dilution ratio of serum, and the ordinate indicates absorbance.

1 Claims:

1. A method of measuring a polynucleotide which is either single stranded as such or a single stranded polynucleotide obtained from a double stranded polynucleotide to be measured, which comprises, contacting the single-stranded polynucleotide to be measured with an immobilised labelled single-stranded polynucleotide which is able to hybridise with said single-stranded polynucleotide in aqueous solution to produce a double-stranded polynucleotide, breaking the double-stranded polynucleotide thereby obtained by means of a double-stranded polynucleotide restriction enzyme, and measuring the amount of labelling substance in the solution obtained and/or on immobilised material, as appropriate as a measure of said single stranded and/or said double stranded polynucleotide to be measured.

2. The method of claim 1, wherein said single-stranded polynucleotide to be measured is a single-stranded deoxyribonucleic acid or a single-stranded ribonucleic acid.

3. The method of claim 1 or 2, wherein said labelling substance is selected from radioisotopes, fluorescent materials, luminescent materials, magnetic materials, enzymes, prosthetic groups thereof, coenzymes and enzyme inhibitors.

4. The method of claim 3, wherein the labelling substance is a large molecule substance and has been introduced into the single-stranded polynucleotide prior to immobilisation thereof.

5. The method of any one of claims 1 to 4, wherein the single-stranded polynucleotide which is labelled has been obtained by denaturation of a double stranded polynucleotide containing the single stranded polynucleotide to be measured.

6. The method of any one of claims 1 to 3, wherein the single stranded polynucleotide which is

1 labelled has been obtained by a solid phase
polynucleotide synthesis method or by genetic synthesis
with γ -DNA using a plasmid.

7. The method of any one of the preceding claims,
5 wherein the carrier material is selected from Sepharose,
Sephadex, cellulose gel, ion exchange resin, filter
paper, nitrocellulose, nylon, polystyrene and
polyacrylamide.

8. The method of any one of the preceding claims,
10 wherein said contacting is carried out at from 20 to 70°C
at a pH of from 5 to 9 over a period of from 0.5 to 40
hours.

9. The method of any preceding claim, wherein the
restriction enzyme is added to the aqueous solution after
15 hybridisation has been effected therein.

10. A kit of reagents for use in the method of
any preceding claim which comprises an immobilised
labelled single stranded polynucleotide which is able to
hybridise with a single-stranded polynucleotide in an
20 aqueous solution to produce a double-stranded
polynucleotide and a restriction enzyme.

25

30

35

1/1

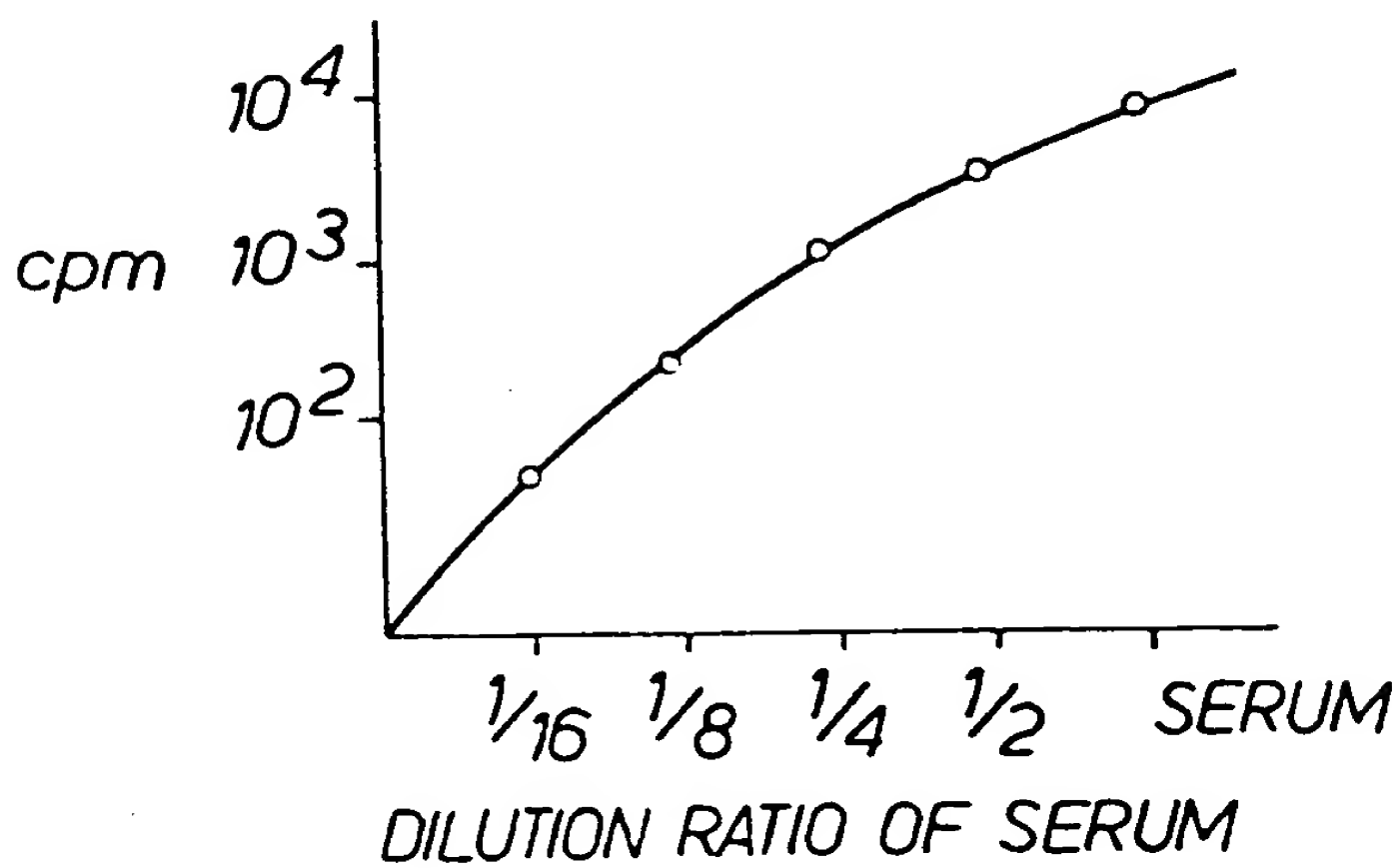


FIG. 1.

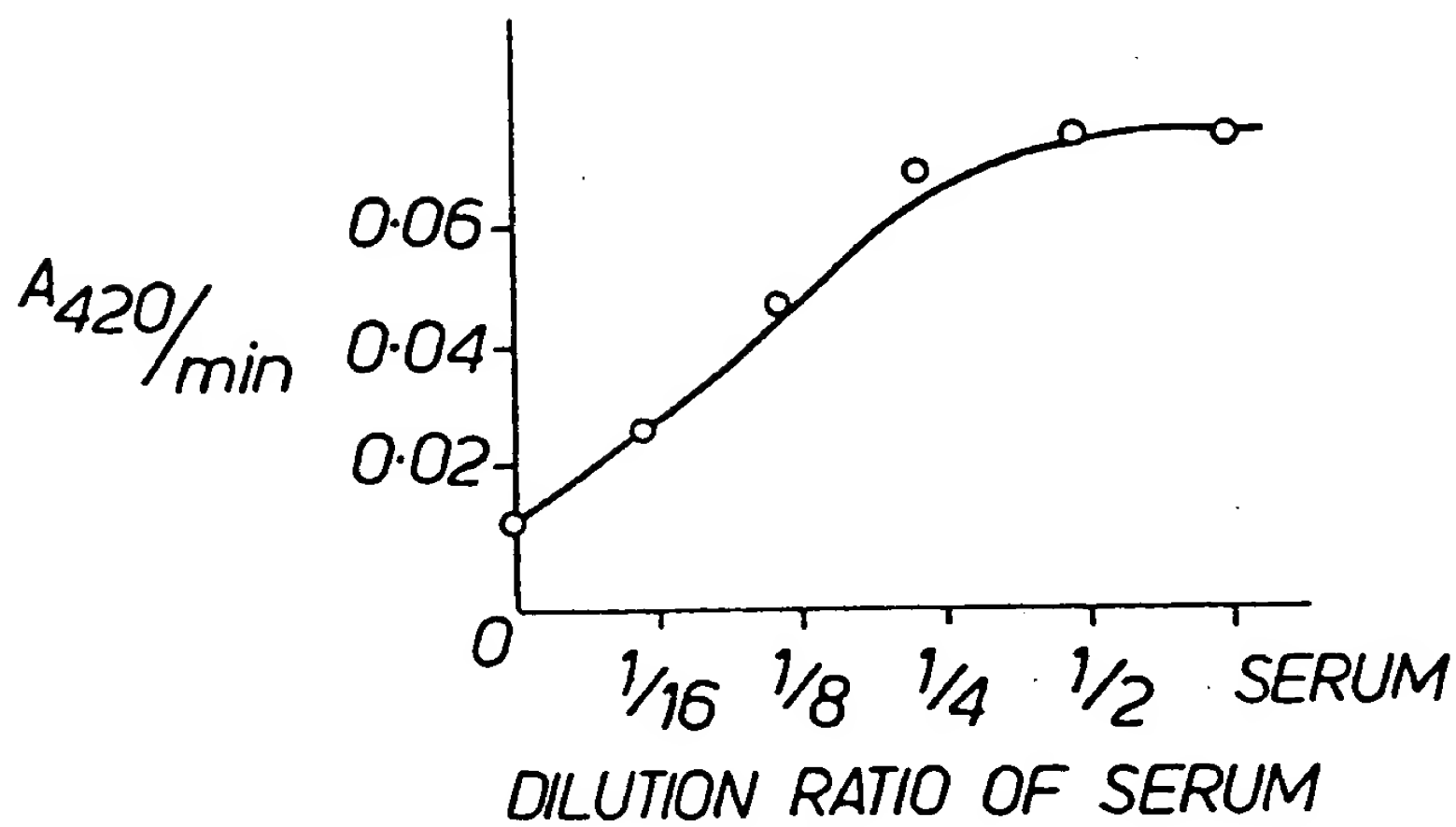


FIG. 2.

12

EUROPEAN PATENT APPLICATION

21 Application number: 84307289.3

51 Int. Cl.³: C 12 Q 1/68

22 Date of filing: 23.10.84

INTERNATIONAL LIBRARY
 SCIENCE RESEARCH INFORMATION SERVICE

30 Priority: 25.10.83 JP 199702/83

43 Date of publication of application:
 22.05.85 Bulletin 85/21

88 Date of deferred publication of search report: 24.02.88

84 Designated Contracting States:
 CH DE FR GB IT LI NL

71 Applicant: FUJIREBIO KABUSHIKI KAISHA also trading
 as FUJIREBIO INC.
 6-7, Shimoochiai 4-chome Shinjuku-ku
 Tokyo 161(JP)

72 Inventor: Ashihara, Yoshihiro
 2-402, Fuchudanchi 28-1, Harumicho, 1-chome
 Fuchu-shi Tokyo(JP)

72 Inventor: Kasahara, Yasushi
 310, 4-4, Nagayama 3-chome
 Tama-shi Tokyo(JP)

74 Representative: Silverman, Warren et al,
 HASELTINE LAKE & CO. Hazlitt House 28 Southampton
 Buildings Chancery Lane
 London WC2A 1AT(GB)

54 Method of measuring polynucleotide and reagent kit for use therein.

57 Disclosed is a method of measuring a polynucleotide
 such as DNA and RNA in a body fluid which does not require
 immobilisation of the test sample and whereby the poly-
 nucleotide is easily determined, wherein an immobilised
 single-stranded polynucleotide which is labelled is hybrid-
 ised with the single-stranded polynucleotide to be measured,
 the double-stranded polynucleotide formed by hybridisation
 is broken by means of a restriction enzyme and the labelling
 substance present is then determined as a measure of the
 polynucleotide being determined.

EP 0 142 299 A3



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	EP-A-0 070 687 (STANDARD OIL CO.) * Page 2, line 23 - page 3, line 18; page 4, lines 4-24; page 6, line 20 - page 7, line 14; page 11, lines 2-30 * ---	1-3,5,7	C 12 Q 1/68
X	US-A-4 351 901 (C.P. BAHL) * Abstract; column 3, lines 21-36; column 5, lines 4-19; column 13, lines 2-30; column 9, example 1 * ---	1-3,8	
E	EP-A-0 123 513 (AMERSHAM INTERNATIONAL) * Page 2, line 29 - page 3, line 18; page 12, lines 20-25; page 18, example 2; page 24, claim 1 * ---	1-3,7-9	
Y	US-A-4 302 204 (G.M. WAHL et al.) * Column 1, line 58 - column 2, line 14; column 2, lines 35-41; column 6, examples I,II; column 9, example 8; column 10, example 10; column 12, lines 18-28 * ---	1-3,5,7 -9	
Y	WO-A-8 301 459 (ORION-YHTYMA OY) * Page 3, lines 12-33; page 8, line 24 - page 9, line 16; page 26, lines 3-24 * ---	1-3,5,7 -9	C 12 Q 1/00 C 07 H 21/00 C 12 N 15/00
Y	US-A-4 358 535 (S. FALKOW et al.) * Column 1, line 58 - column 2, line 17; column 2, lines 32-61; column 4, lines 40-47; column 9, lines 26-58; column 10, lines 3-23 * -----	1-3,5,7 -9	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 25-11-1987	Examiner VAN BOHEMEN C.G.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			